

Baicalein attenuates intimal hyperplasia after rat carotid balloon injury through arresting cell-cycle progression and inhibiting ERK, Akt, and NF- κ B activity in vascular smooth-muscle cells

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Received: 17 March 2008 / Accepted: 25 June 2008 / Published online: 29 July 2008
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Abstract Baicalein (5,6,7-trioxyflavone-7-O-beta-D-glucuronide) derived from the Chinese herb *Scutellaria baicalensis* is well known as a lipoxygenase inhibitor. We investigated baicalein-mediated inhibitory effects on vascular smooth-muscle cell (VSMC) proliferation and intimal hyperplasia by balloon angioplasty in the rat. In vascular injury studies, baicalein significantly suppressed intimal hyperplasia by balloon angioplasty. Baicalein significantly inhibited cell proliferation via a lipoxygenase-independent pathway using [3 H]thymidine incorporation, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), and flow cytometry assays. At the concentrations used, no cytotoxic effect on cell culture was found. Baicalein blocks cell-cycle progression in S/G2/M phase, consistent with the cell-cycle effects, baicalein significantly inhibited cyclin D1, p42/44 mitogen-activated protein kinase (MAPK), and Akt phosphorylation without change in the other cell-cycle regulatory proteins. Furthermore, baicalein attenuated serum-induced deoxyribonucleic acid (DNA) binding ac-

tivity of nuclear factor kappa B (NF- κ B). These results show that baicalein blocks cell proliferation via blocking cell-cycle progression and proliferating events, including p42/44 MAPK and Akt activations as well as NF- κ B activation. It also inhibits intimal hyperplasia after balloon vascular injury in the rat, indicating the therapeutic potential for treating restenosis after arterial injury.

Keywords Baicalein · Proliferation · Vascular smooth-muscle cells · Restenosis

Introduction

The structure of the arterial wall and its vasoactive properties are mainly derived from vascular smooth-muscle cells (VSMCs), which represent a prominent cell type in this tissue. VSMCs are involved in several pathological conditions, such as neointima formation, restenosis after acute vascular injury, and chronic pathological processes such as atherosclerosis (Ross 1993). Efforts to inhibit VSMCs proliferation in vascular injury models, either by modulating cellular mediators of the proliferative response or by interfering with the cell-cycle machinery, have provided insights into neointima formation. Cell-cycle progression is a tightly controlled event regulated by cyclins, cyclin-dependent kinases (CDKs), their substrate proteins, CDK inhibitors, and tumour-suppressor gene products p53 and retinoblastoma proteins (Rb) (Golias et al. 2004).

Cell proliferation is initiated by the transduction of mitogenic signals from the cell-surface receptor to nucleus via an activation cascade, receptor protein tyrosine kinase, p42/44 mitogen-activated protein kinase (MAPK) (Katz et

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al. 2007), and PI3-K/Akt signaling pathway (Mehdi et al. 2007). p42/44 MAPK is a serine/threonine kinase activated by a dual phosphorylation on threonine and tyrosine residues (Zhang and Liu 2002). Phosphorylated and activated p42/44 MAPK translocates into the nucleus, where it phosphorylates several nuclear transcription factors, including nuclear factor kappa B (NF- κ B), ultimately leading to initiation of the gene transcription, transition of cells from a quiescent to a proliferative state, deoxyribonucleic acid (DNA) synthesis, and cell division (Chen et al. 2001a, b; Hoshi et al. 2000). Phosphorylation of the serine/threonine kinase Akt is an important signaling pathway that mediates cell proliferation and survival signals in response to growth factors and cytokines (Manning and Cantley 2007). Akt also signals activation of NF- κ B through phosphorylation and activation of inhibitor-kappa B kinase-alpha (IKK- α) (Adhikari et al. 2006).

NF- κ B has been reported to play a pivotal role in regulating gene expression controlling inflammation, cell differentiation, apoptosis, and proliferation (Courtois and Gilmore 2006). Immunohistochemical studies indicate that human atherosclerotic tissues express NF- κ B proteins (Brand et al. 1996). The expression of NF- κ B is enhanced in vascular tissue during VSMC proliferation after lumen injury (Cercek et al. 1997). These studies indicate that NF- κ B is involved in VSMC proliferation *in vitro* and *in vivo*. Under normal conditions, NF- κ B is present in the cytoplasm as an inactive heterotrimer consisting of p50, p65, and inhibitor κ B (I κ B) subunits. On activation, degradation of I κ B exposes nuclear localization signals on the p50/p65 heterodimer, leading to nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription. I κ B degrades immediately after injury in vascular walls. These findings suggest that I κ B plays a key role in regulating NF- κ B activation in VSMC proliferation of vascular walls (Landry et al. 1997).

Baicalein (5,6,7-trioxyflavone-7-O-beta-D-glucuronide) is a flavonoid from *Scutellaria baicalensis*. Previous studies have shown that baicalein exhibits a multitude of pharmacological activities, including anti-inflammatory (Hong et al. 2002), antiallergic (Kimata et al. 2000), anticancer (Pidgeon et al. 2002; Lee et al. 2005; Chao et al. 2007), and anti-oxidative (Shao et al. 2002) activities. Among them, anti-thrombotic, antiproliferative, and antioxidative effects lead to cardiovascular protective properties. These findings have highlighted the fact that baicalein has the therapeutic potential to treat atherosclerosis and restenosis. To our knowledge, the study reported here is the first to show direct evidence that baicalein inhibits rat VSMC proliferation *in vivo* after arterial injury. We also examined the effect of baicalein on VSMC proliferation induced by serum and focused on transcription factor NF- κ B, cell-cycle progression regulators, p42/44 MAPK, and Akt signaling pathways.

Materials and methods

Restenosis model and histological examination

Wistar rats were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. Vascular injury was induced by balloon-catheter inflation of the rat common carotid artery (Peng et al. 2004). The rats were anesthetized with an intraperitoneal administration of sodium chlorohydrate (37 mg/kg), and a 2-Fr Fogarty arterial embolectomy balloon catheter was inserted into the left carotid artery. The catheter was passed through the entire length of the artery three times to cause the vascular injury. After removal of the catheter from the extracarotid artery, the wound was ligated and closed. Vehicle [0.5% carboxymethylcellulose (CMC)] or baicalein (10 mg/kg per day) were orally administered into the rat 3 days before to 14 days after the vascular injury. The right carotid artery remained untreated and served as the intra-individual control. However, 14 days after injury, rats were euthanatized with intraperitoneal administration of pentobarbital (60 mg/kg), and left and right carotid arteries were removed and fixed with 4% formaldehyde and embedded in paraffin. The embedded tissues were sectioned at 5- μ m thick, stained with hematoxylin–eosin, and analyzed using microscopy.

Cell culture

VSMCs were isolated from 200-g male Wistar rat. Aortic strips were cut into small pieces and placed in six-well culture plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μ g/ml) (Gibco). Strips were incubated at 37°C in a humidified, 5% CO₂ atmosphere for 10 days to allow VSMC migration from the vessel onto the dish. The medium was changed every 3 days, and cells were subcultured by 0.05% trypsin/0.02% ethylenediaminetetraacetate (EDTA) within six passages for the following experiments.

[³H]Thymidine incorporation assay

Confluent VSMCs were trypsinized, suspended in DMEM supplemented with 10% FBS, and seeded at 1.0×10^4 cells per well into 96-well plates. After 24 h, the cells were washed twice with phosphate buffered saline (PBS) and starved with serum-free DMEM for 24 h. Baicalein was dissolved in DMSO, and DMSO was used as vehicle control (final concentration 0.1%) in all *in vitro* experiments. The cells were incubated with or without indicated reagents and 10% FBS for 48 h and then harvested for DNA synthesis detection. Before harvest, cells were incubated with [³H]

thymidine (1 $\mu\text{Ci/ml}$, Amersham Pharmacia) for 16 h, processed and harvested with Filter-Mate (Packard), and incorporated radioactivity was determined.

MTT assay

The alteration of cell number was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. In brief, after treatment of cells with or without the indicated agent and/or serum for 48 h, cells were washed twice with PBS and incubated with 0.5 mg/ml MTT (Sigma) for 4 h. The reagent was reduced by living cells to form an insoluble blue formazan product. After the incubation period, cells were washed with PBS, solubilized with DMSO, and quantified using an enzyme-linked immunosorbent assay (ELISA) reader at the absorbance of 550 nm.

Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) released into cell culture medium is an index of cytotoxicity and evaluates the permeability of the cell membrane. After an incubation of 24 h with various drug concentrations in serum, the culture supernatants were collected. The LDH assay was performed using the CytoTox96 Nonradioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The percentage of LDH released was expressed as a proportion of the LDH released into the medium compared with the total amount of LDH present in cells treated with 2% Triton X-100.

FACScan flow cytometric assay

After cells were treated with vehicle (0.1% DMSO) or baicalein for the indicated times, they were harvested by trypsinization, fixed with 70% (v/v) alcohol at 4°C for 30 min, and washed with PBS. They were then centrifuged and resuspended with 0.5 ml propidium iodide solution containing Triton X-100 (0.1%, v/v), RNase (100 $\mu\text{g/ml}$), and propidium iodide (80 $\mu\text{g/ml}$). DNA content was analyzed with the FACScan and CellQuest software (Becton Dickinson, Mountain View, CA USA).

Protein extraction and Western blot analysis

VSMCs were rendered quiescent by serum starvation for 2 days. After cell treatment with the indicated agent for different time periods [p42/44 MAPK and phosphorylated p42/44 MAPK (Santa Cruz), 10 min; phosphorylated Akt (Upstate Biotechnology), 15 min; phosphorylated I κ B- α , 30 min; I κ B- α , 1 h; cyclins, CDKs, p21, and p27 (Santa Cruz), 24 h], they were washed twice with PBS pH 7.4 and

harvested on ice-cold buffer A containing 20 mM Hepes, pH 7.4, 2 mM EDTA, 1 mM β -glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4 , 1% Triton, 10% glycerol, 1 $\mu\text{g ml}^{-1}$ leupeptin, 1 $\mu\text{g ml}^{-1}$ aprotinin, and 400 μM phenylmethylsulfonyl fluoride. The cell lysate was centrifuged and the supernatant used for Western blot analysis as described previously (Lu et al. 2006). Signal detection was performed with an enhanced chemiluminescence detection kit (Amersham).

Detection of NF- κ B activity

DNA binding activities of NF- κ B were determined using electrophoretic mobility shift assay (EMSA). VSMCs were activated with stimulants for 1 h (NF- κ B) and collected with a cell scraper. Nuclear extracts were prepared and applied to gel shift assay as described previously (Pan et al. 2003). Briefly, 2 μg of nuclear extracts were incubated with a 35-base-pair double-stranded ^{32}P -labeled probe encoding the κB consensus sequence (5'-AGT TGA GGG GAT CCC CCC AGG C-3') in the binding buffer containing 10 mM Tris-HCl, 40 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, 1% deoxycholate, and 3 $\mu\text{g/ml}$ polydeoxyinosinic-deoxycytidylic acid at room temperature for 30 min. Then, samples were applied to native 5% polyacrylamide gels and analyzed on autoradiography. For competition assay, 20-fold molar excess unlabeled consensus oligonucleotide was added 30 min prior to addition of the labeled probe. Components of NF- κ B proteins were identified by supershift assay using antibodies against p65 antibodies.

Statistical analysis

Data are presented as the mean \pm standard error (SE) for the indicated number of separate experiments. Statistical significance was assured by one-way analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons. A P value <0.05 was considered significant statistically.

Results

Baicalein inhibits intimal hyperplasia by balloon angioplasty

Eighteen rats were assigned to orally receive vehicle (0.5% CMC) or baicalein (10 mg/kg per day) beginning 3 days before balloon angioplasty. At day 14 after angioplasty, animals were euthanized to detect vascular injury and intimal thickening. As demonstrated in Fig. 1, the carotid artery without balloon angioplasty exhibited normal vessel

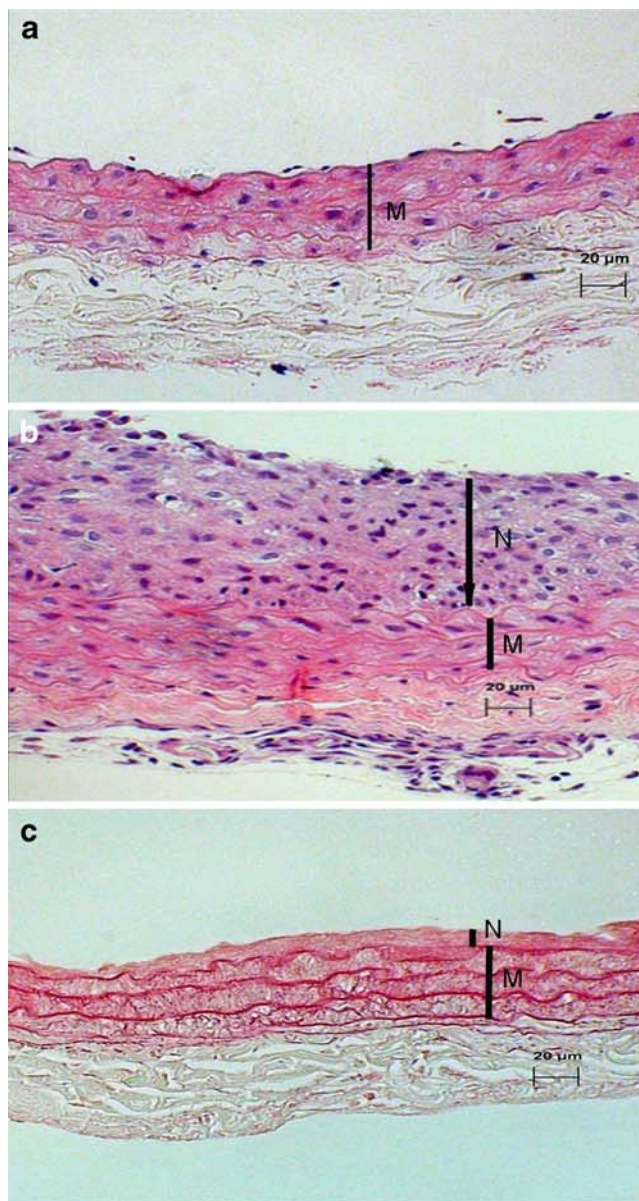


Fig. 1 Responses of rat carotid arteries to balloon injury. Representative observations are sections from uninjured vessel (**a**), balloon-injured vessel (**b**), and baicalein-treated balloon-injured vessel (**c**). All pictures were taken at $\times 200$ magnification. Data are also quantified by the neointima/media ratio of common carotid arteries after balloon injury from each group of animals (**d**). Data are expressed as means \pm standard error of six animals in each group. # $P < 0.01$ and ** $P < 0.01$ compared with basal and control, respectively. (*N* neointima layer, *M* media layer)

thickness in the media layer (Fig. 1a), and the injured vessel showed a profound neointimal thickening (Fig. 1b). However, there was an obvious reduction in neointimal thickness in the section from the baicalein-treated animals (Fig. 1c), and data are also quantified by the neointima/media ratio of common carotid arteries after balloon injury from each group of animal studies (Fig. 1d).

Baicalein inhibits VSMC proliferation

VSMCs proliferate after vascular injury in response to several growth factors and cytokines such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and angiotensin II (Ross 1993; Griendling et al. 1997). In our study, we use 10% FBS instead of a single growth factor to induce cell proliferation. Because FBS contains a range of growth factors, including PDGF, FGF, transforming growth factor, serotonin, and thrombin (Wang et al. 2000), we used FBS to mimic the multiple factors environment *in vivo*. To examine the effect of baicalein on the regulation of cell growth, [^3H]thymidine incorporation and MTT assay were used to determine DNA synthesis and cell number, respectively. As demonstrated in Fig. 2a,b, baicalein inhibited the serum-induced increase of DNA synthesis ($\text{IC}_{50} = 1.97 \times 10^{-5}\text{M}$) and cell number ($\text{IC}_{50} = 2.32 \times 10^{-5}\text{M}$) in a concentration-dependent manner, suggesting the antiproliferative action of baicalein. Furthermore, baicalein showed little influence on the release reaction of lactate dehydrogenase (Fig. 2c), revealing that the antiproliferative action of baicalein was not due to the cytotoxic effect.

Lipoxygenase metabolites do not reverse the action to baicalein

Baicalein is well known as a lipoxygenase inhibitor (Butenko et al. 1993; Sekiya and Okuda 1982). We determined whether the antiproliferative effect of baicalein resulted from lipoxygenase inhibition. Data showed that lipoxygenase metabolites 12- and 15-hydroxyeicosatetraenoic acid (HETE), either alone or combined, could not induce the proliferative effect (Fig. 3a). Furthermore, the combination of 12- and 15-HETE also could not reverse the antiproliferative action to baicalein (Fig. 3a,b), suggesting

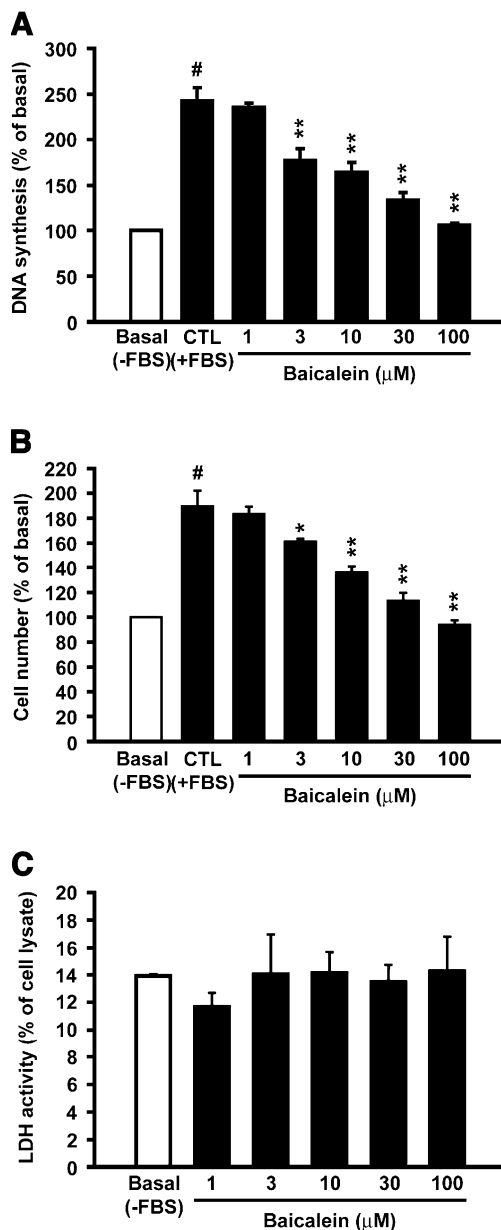


Fig. 2 Effect of baicalein on proliferation and cell number of rat vascular smooth-muscle cells. Cells were rendered quiescent for 24 h, treated with or without baicalein. Then vehicle or fetal bovine serum (FBS) (10%, v/v) was added for 48 h. Sixteen h before termination of the incubation period, [³H]thymidine was added to the cells (a). After the final treatment, cells were harvested to detect [³H]thymidine incorporation. (b), the cell number was detected using MTT assay after the incubation period, as described in [Materials and methods](#). Cells were incubated in the absence or presence of baicalein for 24 h, and then the culture medium was obtained for to detect lactate dehydrogenase (LDH) release reaction, as described in [Materials and methods](#) (c). Data are expressed as mean ± standard error of six determinations. [#]*P*<0.01 compared with basal group; **P*<0.05 and ***P*<0.01 compared with control group

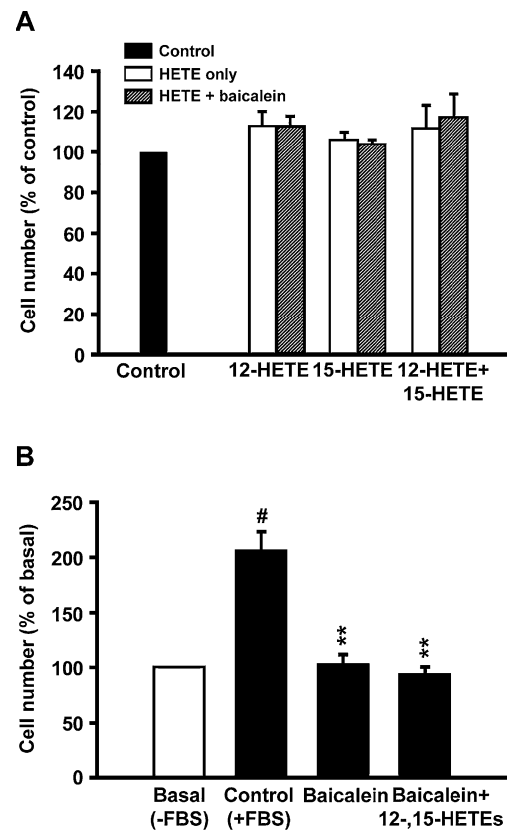


Fig. 3 Effect of lipoxigenase metabolites and baicalein on the cell viability of rat vascular smooth-muscle cells. Cells were rendered quiescent for 48 h, and then treated with or without 12-hydroxyeicosatetraenoic acid (HETE) (100 nM), 15-HETE (100 nM), or baicalein (50 μM) for 48 h (a). Cells were rendered quiescent for 48 h, and then treated with or without 12-HETE (100 nM), 15-HETE (100 nM), or baicalein (50 μM), and then vehicle or fetal bovine serum (FBS) (10% v/v) was added for 48 h (b). After the incubation period, the cell number was detected using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method, as described in the [Materials and methods](#). Data are expressed as mean ± standard error of six determinations. [#]*P*<0.01 compared with basal group; ***P*<0.01 compared with control group

that the antiproliferative effect of baicalein was not via the inhibition of cellular lipoxigenase.

Baicalein inhibits cell-cycle progression and related protein expression

To determine whether baicalein influences the cell-cycle machinery, we examined cell-cycle progression using flow cytometric assessment. We found that about 96% of cells were arrested in the G0/G1 phase of the cell cycle when the cells were rendered quiescent by the deprivation of serum. After supplementation with 10% FBS for 24 h, a significant increase of S and G2/M phases of the cell cycle was observed (2.9-fold compared with the basal of 1-fold); however, this effect was completely abolished by 50 μM baicalein (Fig. 4a). We next examined the cell-cycle

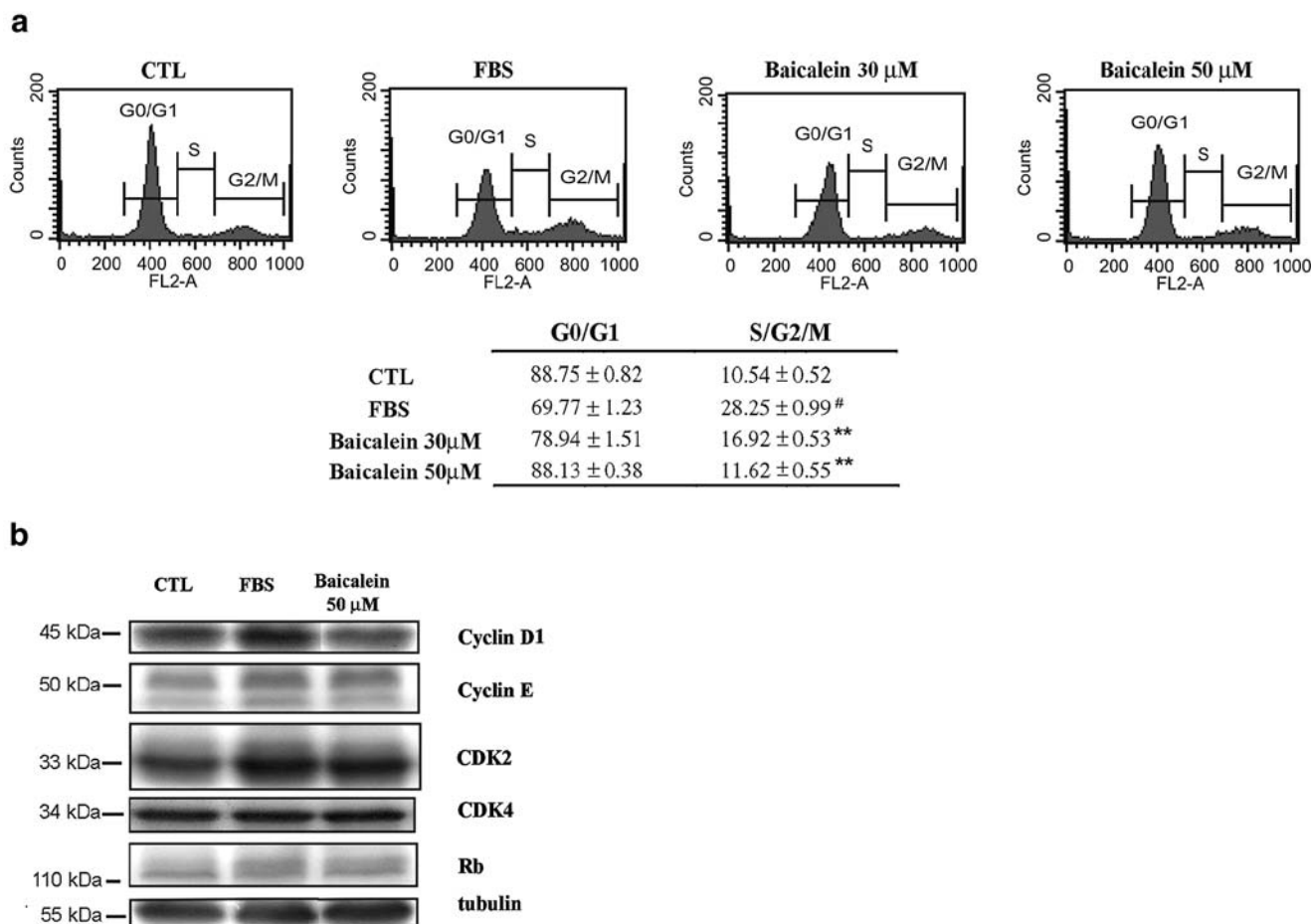


Fig. 4 Regulation of cell-cycle progression and regulatory proteins by baicalein. Cells were rendered quiescent for 48 h, and then fetal bovine serum (FBS) was added for 24 h in the absence or presence of baicalein (30 μ M and 50 μ M). Cells were then harvested to detect cell-cycle progression using flow cytometric methods (a) and cell-

cycle regulatory proteins expression using the Western blot method (b). Data are expressed as mean \pm standard error of four determinations. [#] $P < 0.01$ compared with basal group; ^{**} $P < 0.01$ compared with control group

regulatory proteins, which represent the entering of the cell cycle from G0/G1 to S phase. Baicalein significantly inhibited cyclin D1 without change in cyclin E expression, (CDK)-2 and 4, and retinoblastoma protein (Rb) proteins. Data showed a profound increase of cyclin D1 expression after stimulation of cells with serum; baicalein completely abolished this serum action (Fig. 4b). These results suggest that baicalein could effectively inhibit serum-induced cell-cycle progression and retain the cells in the quiescent state.

Baicalein inhibits phosphorylation of p42/44 MAPKs and Akt/PKB

We measured the effect of baicalein on serum-induced activation of p42/44 MAPKs, which act downstream of several types of mitogenic stimulation and upstream of several transcriptional events. The phosphorylated p42/44 MAPKs were examined as the determination of MAPK

activation. Data showed that serum induced MAPK phosphorylation and therefore kinase activity. Both baicalein and PD98059, a selective MAPK inhibitor, significantly inhibited the effects of serum addition (Fig. 5a), revealing that baicalein behaves as an upstream regulator of p42/44 MAPK activation. In addition, PI 3-kinase is a ubiquitously expressed enzyme family that plays a crucial role in the regulation of several cellular functions, including cell proliferation. To determine the effect of baicalein and PD98059 on the PI 3-kinase-signaling pathway, we measured their action on Akt/protein kinase B (PKB) phosphorylation, which is downstream of PI 3-kinase occurring in response to growth stimuli and cell-survival signaling pathway. As demonstrated in Fig. 5b, serum induced a marked increase of Akt/PKB phosphorylation that was completely inhibited by baicalein and LY294002 (10 μ M), a selective inhibitor of PI 3-kinase activation. However, PD98059 significantly increased rather than

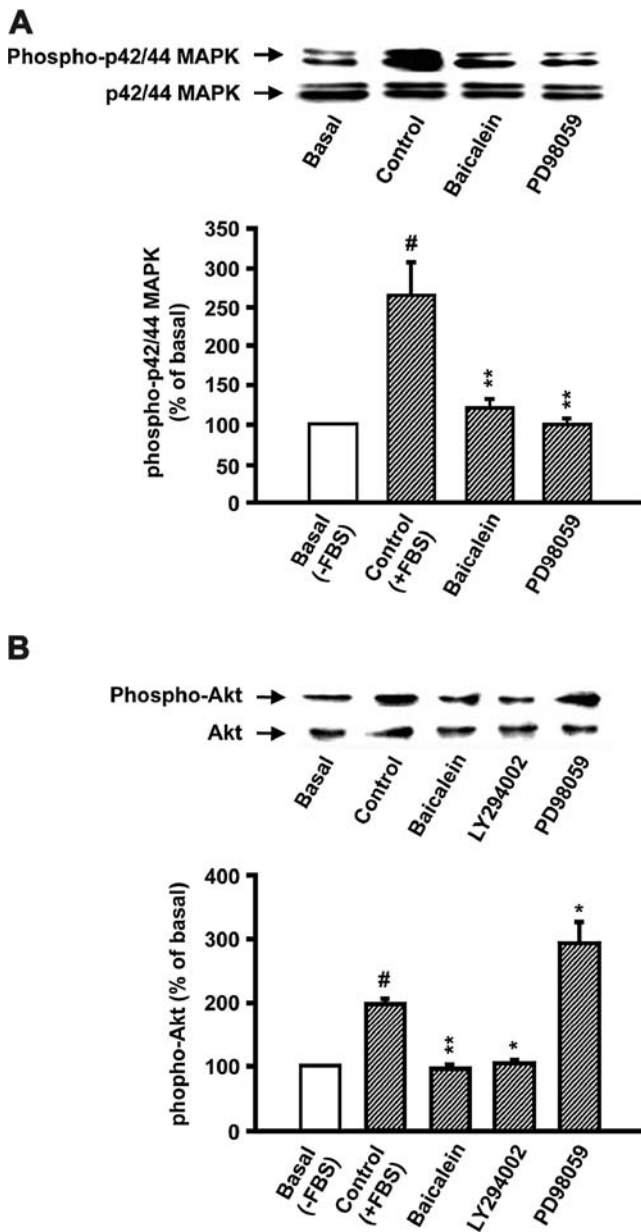


Fig. 5 Effect of baicalein and PD98059 on p42/44 mitogen-activated protein kinase (MAPK) and Akt/protein kinase B (PKB) phosphorylation in rat vascular smooth-muscle cells. Cells were rendered quiescent for 24 h then incubated in the absence or presence of baicalein (50 μ M), or PD98059 (10 μ M), or LY294002 (10 μ M) for 1 h, and vehicle or fetal bovine serum was added to the cells for 10 min. Cells were harvested for to detect phosphorylated-p42/44 MAPK and p42/44 MAPK (a) and phosphorylated-Akt/PKB and Akt (b) using Western blot detection, as described in **Materials and methods**. Data are expressed as mean \pm standard error of four determinations. # P <0.01 compared with basal group; * P <0.05 and ** P <0.01 compared with control group

decreased the amount of phosphorylated Akt/PKB (Fig. 5b). Thus, baicalein and LY294002 exhibited the inhibitory and PD98059 the stimulatory effect on serum-induced PI 3-kinase activation.

Baicalein blocks serum-mediated I κ B- α degradation and NF- κ B activation

It is well established that the NF- κ B activity is regulated by I κ B proteins and that phosphorylation and degradation of I κ B- α results in activation of NF- κ B. As shown in Fig. 6a,

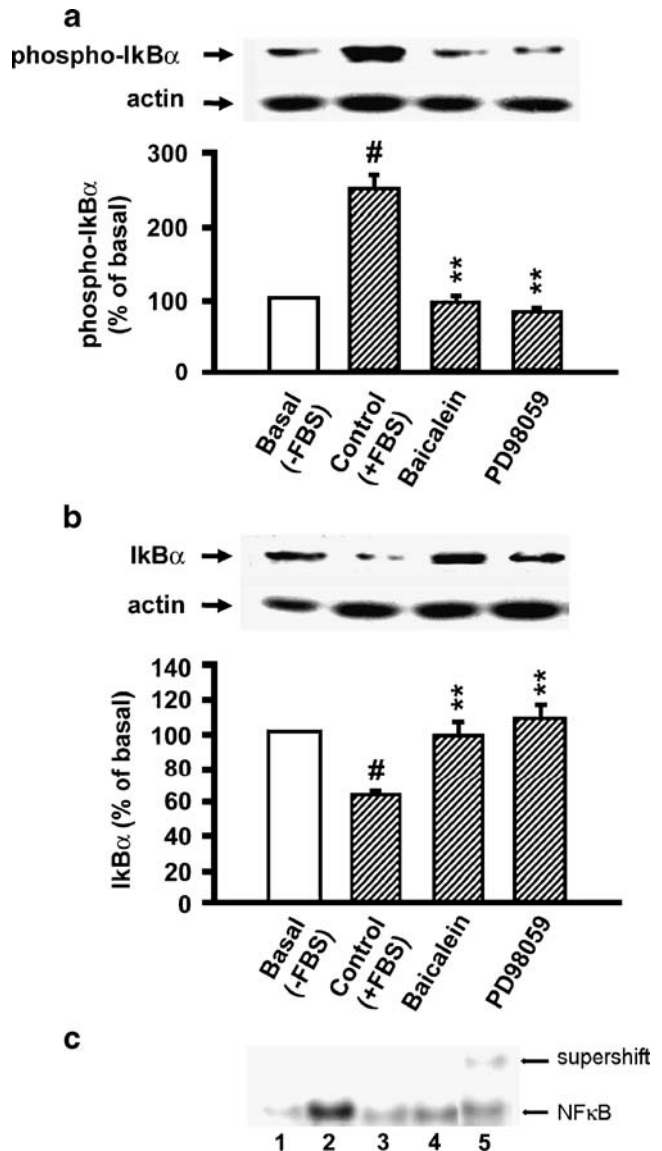


Fig. 6 Effect of baicalein and PD98059 on phosphorylation (a) and degradation (b) of inhibitor kappa B alpha (I κ B- α) and nuclear factor kappa B (NF- κ B) deoxyribonucleic acid (DNA) binding activity (c). Cells were rendered quiescent for 24 h, treated with or without the indicated agent for 1 h, and then vehicle or fetal bovine serum (FBS) was added for 10 min (a) or 1 h (b, c). After treatment, total cellular proteins were extracted to detect I κ B- α phosphorylation and degradation using immunoblot by specific antibodies (a, b). The nuclei were extracted, and electrophoretic mobility shift assay was performed to detect the DNA binding activity of NF- κ B (1 basal, 2 FBS control, 3 baicalein, 4 PD98059, 5 p65 antibody), as described in **Materials and methods**. Data are expressed as mean \pm standard error of three determinations. # P <0.01 and ** P <0.01 compared with basal and control, respectively

exposure of quiescent cells to serum for 10 min stimulated a profound increase of I κ B- α phosphorylation; both baicalein and PD98059 (30 μ M) significantly inhibited the serum-induced effect. Additionally, the treatment of quiescent cells with serum for 60 min evoked a marked degradation of I κ B- α . This serum action also completely reversed in the presence of baicalein or PD98059 (Fig. 6b). Furthermore, we determined the DNA binding activity of NF- κ B by EMSA using an oligonucleotide containing NF- κ B consensus sequence. Data showed that both baicalein and PD98059 effectively inhibited serum-induced DNA binding activity of NF- κ B (Fig. 6c).

Discussion

Herbal medicines used by ancient people for thousands of years are now being manufactured in many countries as drugs with standardized quality and quantities of ingredients. Baicalein, one of the major flavonoids contained in the dried roots of *S. baicalensis*, which is widely used in traditional Chinese medicine as a treatment for inflammation, fever, and allergic diseases (Lai et al. 2003). In our laboratory, we have examined many of purified compounds and crude extracts from herbal medicines of Chinese medicinal prescriptions to find out their active component against VSMCs proliferation (Pan et al. 2003; Guh et al. 1996), as it is a causative factor in several vascular diseases, such as atherosclerosis and restenosis after acute vascular injury.

The *in vivo* animal model of balloon injury has been the most frequently used in restenosis study. Rats are regularly used because of the reproducible major formation of intimal hyperplasia within 2 weeks after balloon injury (Clowes and Schwartz 1985). Our data showed for the first time that baicalein has an *in vivo* antiproliferation activity to block intimal thickening in the rat model. When baicalein was administrated orally, the conjugated metabolite appeared in the bloodstream within 10 min, indicating very rapid absorption and simultaneous glucuronidation/sulfation. When compared with intravenous bolus administration with dose correction, the absolute absorption was 40% (Lai et al. 2003).

As baicalein effectively inhibited neointima formation after balloon injury, the *in vivo* effect would be carried out to examine its therapeutic mechanism. Although baicalein has been reported to have *in vitro* antiproliferative activity in VSMCs (Huang et al. 1994; Dethlefsen et al. 1994; Natarajan et al. 1994; Nishio and Watanabe 1997; Preston et al. 2006), the exact molecular mechanisms are not known. In our study, baicalein exhibited a marked antiproliferative effect in VSMCs using [3 H]thymidine incorporation, MTT assay, and flow cytometric assessment.

We tested the hypothesis that baicalein could inhibit VSMC proliferation as it did in endothelial cells or tumor cells. There are two reasons indicating that the antiproliferative effects of baicalein is not contributed to the inhibition of lipoxygenase. One is that the IC $_{50}$ value of the antiproliferative effect (about 10 μ mol level) is 30–100 times that of lipoxygenase inhibition (about 0.3 μ M). The other is that lipoxygenase metabolites 12- and 15-HETE, either alone or in combination, could not induce the proliferative effect. They also could not reverse the antiproliferative action of baicalein, suggesting that the antiproliferative effect of baicalein was not via cellular lipoxygenase inhibition.

It has been suggested that several second messengers could inhibit VSMC proliferation, such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), and some prostaglandins (Hayashi et al. 2000; Kronemann et al. 1999). In our study, baicalein could not effectively induce the formation of cAMP and cGMP, and the exposure of 10 μ M indomethacin (a potent cyclooxygenase inhibitor) did not influence the antiproliferative action of baicalein (data not shown). This indicates that the antiproliferative effect of baicalein is not simply through generation of the above second messengers. Furthermore, reactive oxygen species (ROS) are suggested to regulate the cell proliferative effect (Kamata and Hirata 1999), and baicalein was found to exhibit the free radical scavenging activity in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (about 90% scavenging activity). However, in our unpublished data, serum induced little generation of ROS. This rules out the possibility that the free radical scavenging activity of baicalein mediates its antiproliferative action.

Cell-cycle control is a highly regulated process that involves a complex cascade of events. Modulation of the expression and function of cell-cycle regulatory proteins provides an important mechanism for inhibiting cell growth (Nigg 1995). Several reports have shown that baicalein caused an apparent accumulation of prostate cancer cells in the G1 phase (Chen et al. 2001a, b), increased S phase in lung cancer cells (Lee et al. 2005), and elevated G2/M phases in bladder and hepatoma cancer cell lines (Chao et al. 2007; Chang et al. 2002). Our results indicated that baicalein suppressed serum-induced VSMC proliferation and blocked serum-induced progression of the VSMC cell cycle from the G1 to S phase. Data from primary VSMCs indicate that cyclin D1 plays a regulatory role in progression of the cell cycle at the G1/S transition. In this study, we found that the levels of cyclin D1 protein but not other cell-cycle regulatory proteins, such as cyclin E, CDK-2, CDK-4, and Rb, in serum-induced VSMCs were decreased by baicalein. We suggest that the inhibitory effect of baicalein on VSMC proliferation is related to the arrest of cell-cycle progression.

It has been suggested that MAPK-mediated signaling regulates cell-cycle progression induced by growth factors. Nofer and the colleagues reported that in response to stimulation, increased protein expressions of cyclin D1 and cyclin E were also induced in VSMC (Nofer et al. 2001). In our study, both baicalein and PD98059 significantly inhibited MAPK activation. However, only baicalein profoundly reduced cyclin D1 expression, indicating that there existed an undefined modulator, which was regulated differently by baicalein and PD98059.

Additionally, MAPK activation induced by growth factors also causes the translocation of NF- κ B to the nucleus (Hoshi et al 2000). This early event, within the first 2 h, regulates the cell proliferative effect in VSMCs. In our study, we determined the effect of baicalein and PD98059 on serum-induced NF- κ B activation. Initially, we examined their effects on I κ B- α phosphorylation and its following degradation, as these two events are essential for nuclear translocation and activation of NF- κ B. Both baicalein and PD98059 significantly inhibited serum-induced I κ B- α phosphorylation and degradation and therefore blocked NF- κ B nuclear translocation. There are several lines of evidence suggesting that p42/44 MAPK plays a role in NF- κ B activity regulation in VSMCs (Hoshi et al. 2000). The blockade of NF- κ B-related cascade might be explained by the inhibition of MAPK-mediated signaling in our work.

Recently, it was suggested that the PI 3-kinase-mediated pathway plays a key role in cyclin D1 expression and entrance of the S phase in a cell cycle (Gille and Downward 1999). Furthermore, PI 3-kinase-activated Akt/PKB phosphorylation is sufficient to induce E2F transcriptional activity, and inhibition of PI 3-kinase inhibits the cyclin/CDK-mediated pathway (Brennan et al. 1997). This reveals the significance of PI 3-kinase/PKB-involved signaling on the regulation of the cell-cycle machinery. Our data showed that baicalein other than PD98059 significantly inhibited serum-induced activation of PI 3-kinase, explaining its distinct regulation on cyclin D1 expression. Moreover, PD98059 exhibited a complete blockade of the MAPK pathway and then directed serum stimulation to the PI 3-kinase-mediated pathway, resulting in the increase of Akt/PKB phosphorylation (Fig. 5).

In conclusion, we suggest that baicalein exhibits an antiproliferative effect to serum action via inhibition of p42/44 MAPK, NF- κ B transcription factor, PI 3-kinase, and cell-cycle machinery. Baicalein also inhibits intimal hyperplasia after balloon vascular injury in the rat, indicating the therapeutic potential for treating restenosis after arterial injury.

Acknowledgments This work was supported by research grants of the National Science Council of the Republic of China (NSC-96-2628-B002-109-MY3 and NSC96-2321-B-002-031-MY2).

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